

## *Aquabacterium fontiphilum* sp. nov., isolated from spring water

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A short-rod-shaped, Gram-negative, motile bacterial strain, designated CS-6<sup>T</sup>, was isolated from a water sample collected from a spring located inside Nature Valley, Hsinchu County, Taiwan, and was characterized using a polyphasic approach. Phylogenetic analyses based on 16S rRNA gene sequences showed that the strain formed a monophyletic branch at the periphery of the evolutionary radiation occupied by the genus *Aquabacterium* in the class *Betaproteobacteria*. The closest neighbours were *Aquabacterium parvum* B6<sup>T</sup> (96.7% sequence similarity), *Aquabacterium commune* B8<sup>T</sup> (96.6%) and *Aquabacterium citratiphilum* B4<sup>T</sup> (95.9%). The predominant fatty acids were 18:1 $\omega$ 7c (30.5%), 16:0 (27.9%) and summed feature 3 (16:1 $\omega$ 7c and/or iso-15:0 2-OH) (22.7%). The DNA–DNA relatedness of the strain with respect to recognized species of the genus *Aquabacterium* was less than 70%. The isolate was also distinguishable from members of the genus *Aquabacterium* on the basis of phenotypic and biochemical characteristics. It is evident from the genotypic, chemotaxonomic and phenotypic data, therefore, that strain CS-6<sup>T</sup> represents a novel species of the genus *Aquabacterium*, for which the name *Aquabacterium fontiphilum* sp. nov. is proposed. The type strain is CS-6<sup>T</sup> (=LMG 24215<sup>T</sup>=BCRC 17729<sup>T</sup>).

The genus *Aquabacterium*, proposed by Kalmbach *et al.* (1999), currently comprises three species with validly published names: *Aquabacterium citratiphilum*, *A. commune* and *A. parvum*. The genus *Aquabacterium* is a member of the order *Burkholderiales* in the class *Betaproteobacteria* and encompasses Gram-negative, non-spore-forming, motile rods. The type strains of all three species were isolated from biofilms from the Berlin drinking water system (Kalmbach *et al.*, 1999).

The aim of the present study was to determine the taxonomic position of a bacterial strain (designated CS-6<sup>T</sup>), which formed a semi-transparent colony on R2A agar (BD Difco) that had been incubated at 25 °C for 3 days, following inoculation with a water sample collected from a freshwater spring located in Hsinchu County, Taiwan. The type strains of *A. parvum* (B6<sup>T</sup>), *A. commune* (B8<sup>T</sup>) and *A. citratiphilum* (B4<sup>T</sup>) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and were used as reference strains for comparison. Strain CS-6<sup>T</sup> was subjected to a polyphasic taxonomic study.

Subculturing of strain CS-6<sup>T</sup> was performed on R2A agar or a modified version of R2A agar [prepared according to the formula of BD Difco but containing 0.1% (v/v) Tween 80 (Sigma) in place of starch] and incubated at 25 °C for between 48 and 72 h. Strain CS-6<sup>T</sup> was preserved by lyophilization with 20% (w/v) skimmed milk.

To ascertain the morphology of strain CS-6<sup>T</sup>, cells were observed using phase-contrast microscopy (DM 2000; Leica) in the lag, exponential and stationary phases of growth. Cell motility was tested using the hanging drop method. The Spot Test flagella stain (BD Difco) was used to stain any flagella that might be present. A Gram-stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to ascertain the Gram reaction of strain CS-6<sup>T</sup>. Accumulation of poly- $\beta$ -hydroxybutyrate granules was investigated using light microscopy after staining of the cells with Sudan black. Colony morphology was examined using a stereoscopic microscope (SMZ 800; Nikon). The pH range for growth was examined in R2A broth with appropriate biological buffers (pH 4–10, using increments of 0.5 pH units) (Chung *et al.*, 1995). Tolerance of various NaCl concentrations was tested in R2A broth prepared according to the formula of BD Difco medium, except that the NaCl concentration was altered as required

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CS-6<sup>T</sup> is EF626687.

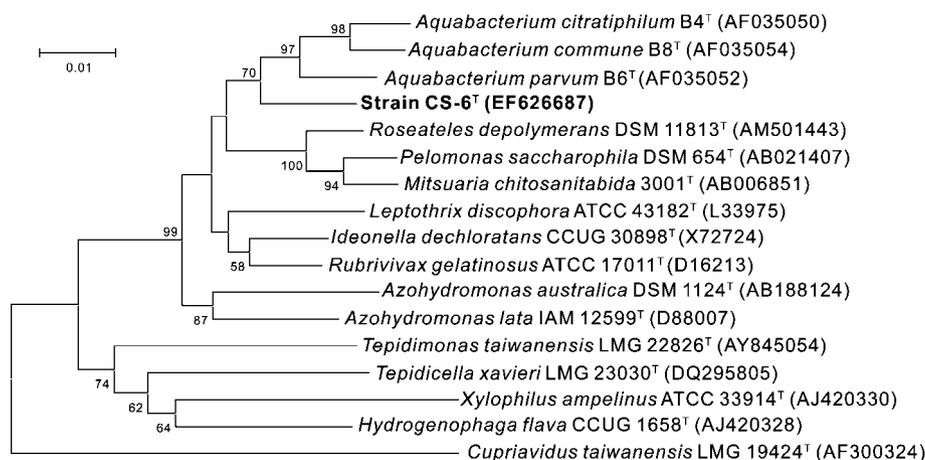
(0, 0.5 and 1.0–10%, w/v, using increments of 1.0%). Growth at various temperatures (4–45 °C) was measured in R2A broth. Cellular growth was determined by measuring the turbidity (OD<sub>600</sub>) of cultures grown at various pH values, NaCl concentrations and temperatures. Anaerobic cultivation was performed on R2A agar or modified R2A agar, using the Oxoid AnaeroGen system.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chen *et al.*, 2001). Sequence analysis was performed using a DNA sequencer (ABI Prism 310; Applied Biosystems) and sequence assembly was achieved by using the Fragment Assembly System program from the Wisconsin package (version 8.1) (GCG, 1995). An almost-complete 16S rRNA gene sequence (1439 nt) from strain CS-6<sup>T</sup> was compared with 16S rRNA gene sequences available from the Ribosomal Database Project and GenBank databases. Multiple sequence alignment for strain CS-6<sup>T</sup> and its closest relatives was performed using BioEdit software (Hall, 1999) and MEGA, version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm by using the distance model of Jukes & Cantor (1969); bootstrap analysis for the neighbour-joining tree was performed (on the basis of 1000 resamplings) using MEGA, version 3.1 (Kumar *et al.*, 2004). Comparisons of the 16S rRNA gene sequence of strain CS-6<sup>T</sup> with sequences of members of the genera in the class *Betaproteobacteria* showed that the strain formed a distinct lineage within the evolutionary radiation of the genus *Aquabacterium* (Fig. 1), a phyletic line that was supported

by all of the tree-making algorithms. The 16S rRNA gene sequence similarity between strain CS-6<sup>T</sup> and the type strains of the members of the genus *Aquabacterium* ranged from 95.9 to 96.7%. It is also apparent from Fig. 1 that the isolate was loosely associated with *A. parvum* B6<sup>T</sup> (96.7% similarity), *A. commune* B8<sup>T</sup> (96.6% similarity) and *A. citratiphilum* B4<sup>T</sup> (95.9% similarity), although these relationships did not have a high level of bootstrap support (78%). No other *Betaproteobacteria* species with validly published names showed more than 96.3% 16S rRNA gene sequence similarity. It is clear that the divergence values observed with respect to all of the recognized *Aquabacterium* species (>3%) were consistent with separate species status (Stackebrandt & Goebel, 1994) for strain CS-6<sup>T</sup>. Further evidence for the separate species status of the strain CS-6<sup>T</sup> comes from phenotypic data.

Whole-genome DNA–DNA hybridization experiments were performed using photobiotin-labelled probes, as described by Ezaki *et al.* (1989). DNA–DNA relatedness was calculated from triplicate experiments. Strain CS-6<sup>T</sup> showed 34 ± 5, 23 ± 5 and 20 ± 3% (means ± SEM) DNA–DNA relatedness with respect to *A. parvum* B6<sup>T</sup>, *A. citratiphilum* B4<sup>T</sup> and *A. commune* B8<sup>T</sup>, respectively.

Chemosystematic studies were carried out to establish whether strain CS-6<sup>T</sup> had a chemical profile consistent with its assignment to the genus *Aquabacterium*. Biomass of strain CS-6<sup>T</sup> and of type strains of species in the genus *Aquabacterium* was obtained after growing the strains on modified R2A medium at 25 °C for 3 days. Fatty acid methyl esters were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI) (Sasser, 1990). The DNA G+C content (mol%) of strain CS-6<sup>T</sup>



**Fig. 1.** Phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strain CS-6<sup>T</sup> and related taxa in the *Betaproteobacteria*. The neighbour-joining method was employed, with the distance model of Jukes & Cantor (1969). The sequence of *Cupriavidus taiwanensis* LMG 19424<sup>T</sup> was used as an outgroup. Numbers at nodes are bootstrap percentages (based on 1000 resamplings); only values above 50% are shown. Bar, 0.01 nucleotide substitutions per nucleotide position.

was estimated (in duplicate) as described by Mesbah *et al.* (1989). The nucleoside mixture was separated by means of HPLC. The predominant fatty acid constituents of strain CS-6<sup>T</sup> were summed feature 3 (16:1 $\omega$ 7c and/or iso-15:0 2-OH), 16:0 and 18:1 $\omega$ 7c. The fatty acid profile of strain CS-6<sup>T</sup> was generally comparable with those of *A. citratiphilum* B4<sup>T</sup>, *A. parvum* B6<sup>T</sup> and *A. commune* B8<sup>T</sup>, but differed in the proportions of certain fatty acids (Table 1). The DNA G+C content of strain CS-6<sup>T</sup> was 63.4  $\pm$  1.0 mol% (mean  $\pm$  SEM), which is consistent with the range of values reported previously for *Aquabacterium* species (65–66 mol%) (Kalmbach *et al.*, 1999).

Additional biochemical tests were performed using the API ZYM, API 20NE (both from bioMérieux) and Biolog GN2 microtest systems according to the methods outlined by the manufacturers. The potential utilization of various electron acceptors [KNO<sub>3</sub>, KNO<sub>2</sub>, NaClO<sub>3</sub>, Na<sub>2</sub>SO<sub>4</sub> or iron(III) citrate] was studied as described by Kalmbach *et al.* (1999). Catalase, oxidase, arginine dihydrolase, urease and lipase (corn oil) activities, nitrate reduction and hydrolysis of starch, casein and Tweens 20, 40, 60 and 80 were determined using standard methods (Gerhardt *et al.*, 1994; Lányi, 1987; MacFaddin, 2000). DNase test agar (BD Difco) was used to assay DNase activity. Sensitivity to

antibiotics was examined by spreading cells (0.5 McFarland standard) on R2A agar and applying discs containing the following antibiotics ( $\mu$ g): ampicillin (10), chloramphenicol (30), gentamicin (10), kanamycin (30), nalidixic acid (30), novobiocin (30), rifampicin (5), penicillin G (10), streptomycin (10), tetracycline (30) and sulfamethoxazole (23.75) plus trimethoprim (1.25). The effects of the various antibiotics on cell growth were assessed after 3 days incubation: susceptibility was scored on the basis of the distance from the edge of the clear zone to the disc. Detailed results from the phenotypic and biochemical analyses of strain CS-6<sup>T</sup> are provided in Table 2 and in the species description. Phenotypic characteristics that serve to differentiate strain CS-6<sup>T</sup> from its closest relatives are presented in Table 2.

From the above data, it is evident that strain CS-6<sup>T</sup> exhibits an overall fatty acid profile that is consistent with those of members of the genus *Aquabacterium* (Table 1). A phylogenetic analysis based on 16S rRNA gene sequencing confirmed the provisional assignment of strain CS-6<sup>T</sup> to the genus *Aquabacterium*, clearly demonstrating that the strain represents a novel subline. Biochemically, isolate CS-6<sup>T</sup> can be differentiated from the most closely phylogenetically and biochemically related species of the genus *Aquabacterium* (Table 2). On the basis of phenotypic, chemotaxonomic and phylogenetic evidence, therefore, strain CS-6<sup>T</sup> represents a novel species within the genus *Aquabacterium*, for which the name *Aquabacterium fontiphilum* sp. nov. is proposed.

**Table 1.** Fatty acid composition (%) of strain CS-6<sup>T</sup> (*A. fontiphilum* sp. nov.) and the type strains of recognized species of the genus *Aquabacterium*

Data for *A. citratiphilum* B4<sup>T</sup>, *A. parvum* B6<sup>T</sup> and *A. commune* B8<sup>T</sup> were obtained in this study. –, Fatty acids constituting <1.0% for all strains.

Fatty acid	Strain CS-6 <sup>T</sup>	<i>A. citratiphilum</i> B4 <sup>T</sup>	<i>A. parvum</i> B6 <sup>T</sup>	<i>A. commune</i> B8 <sup>T</sup>
Straight-chain fatty acids				
10:0	–	1.7	–	–
12:0	7.0	2.3	3.6	4.7
14:0	–	1.3	–	–
16:0	27.9	36.5	20.1	21.3
18:0	–	–	9.1	2.0
Unsaturated fatty acids				
18:1 $\omega$ 7c	30.5	16.0	13.4	14.0
18:1 $\omega$ 9c	2.0	1.6	1.6	–
11-Methyl 18:1 $\omega$ 7c	1.2	–	–	–
Hydroxy fatty acids				
10:0 3-OH	6.5	5.9	5.4	5.1
12:0 2-OH	–	2.9	2.1	1.6
Summed feature 3*	22.7	31.9	44.0	49.5

\*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 comprises 16:1 $\omega$ 7c and/or iso-15:0 2-OH.

**Description of *Aquabacterium fontiphilum* sp. nov.**

*Aquabacterium fontiphilum* [fon.ti.phi'lum. L. n. *fons*, *fontis* a spring, fountain; N.L. neut. adj. *philum* (from Gr. neut. adj. *philon*) loving; N.L. neut. adj. *fontiphilum* loving spring-water environment].

Cells are Gram-negative, non-spore-forming rods (0.5–0.8  $\mu$ m wide and 1.0–2.0  $\mu$ m long) that are motile by means of single polar flagella. Colonies on R2A agar are round, elevated with a knob-like protuberance, semi-transparent and have irregular edges. Colonies are approximately 1.3–1.5 mm in diameter on R2A agar after 48 h incubation at 25 °C. Growth occurs at 15, 25, 30, 37 and 42 °C, in 0–2% NaCl and at pH 5–9. Optimum growth occurs at 25–30 °C, 1.0% NaCl and pH 7.0–8.0. Positive for cytochrome oxidase and weakly positive for catalase. Growth is observed under aerobic and micro-aerophilic conditions. Positive for hydrolysis of urea, gelatin and Tweens 20, 40, 60 and 80. Negative for DNase, lipase (corn oil) and for hydrolysis of skimmed milk, starch and aesculin. Nitrate serves as an alternative electron acceptor, but nitrite, chlorate, sulfate or iron(III) do not. Positive (API ZYM) for alkaline phosphatase, esterase (C4), lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase and naphthol-AS-BI-phosphohydrolase, but negative for trypsin,

**Table 2.** Genotypic and phenotypic characteristics that distinguish strain CS-6<sup>T</sup> from recognized members of the genus *Aquabacterium*

All of the results for strain CS-6<sup>T</sup> were from this study. Data for *A. citratiphilum* B4<sup>T</sup>, *A. parvum* B6<sup>T</sup> and *A. commune* B8<sup>T</sup> were obtained in the present study, with the exception of the DNA G+C contents and details of the isolation sources, which were taken from Kalmbach *et al.* (1999). +, Positive response; -, negative response; w, weak reaction.

Characteristic	Strain CS-6 <sup>T</sup>	<i>A. citratiphilum</i> B4 <sup>T</sup>	<i>A. parvum</i> B6 <sup>T</sup>	<i>A. commune</i> B8 <sup>T</sup>
Isolation source	Spring water	Drinking water	Drinking water	Drinking water
Cell size (µm)	0.5–0.8 × 1–2	0.5 × 2–4	0.5 × 1–2	0.5 × 2–4
Growth on/in:				
R2A agar	+	+	w	w
R2A broth	+	+	–	–
Nutrient agar	w	w	–	–
Hydrolysis of:				
Urea	+	+	+	–
Casein	–	–	–	+
Gelatin	+	+	–	–
Arginine dihydrolase	–	+	–	+
Alkaline phosphatase	+	+	–	+
Lipase (C14)	+	+	–	–
Valine arylamidase	+	–	–	–
Cystine arylamidase	+	–	–	w
α-Chymotrypsin	–	+	–	–
Naphthol-AS-BI-phosphohydrolase	+	–	+	–
Assimilation of:				
i-Erythritol	+	–	–	–
Pyruvic acid methyl ester	–	+	–	–
Succinic acid monomethyl ester	–	+	–	+
<i>cis</i> -Aconitic acid	+	–	–	–
Citric acid	+	–	–	–
Formic acid	+	–	–	–
γ-Hydroxybutyric acid	–	+	–	–
Sebacic acid	–	+	+	–
DNA G+C content (mol%)	63.4	66	65	66

α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, *N*-acetylglucosaminidase, α-mannosidase and α-fucosidase. The following compounds are oxidized in the Biolog GN2 test system: Tween 40, Tween 80, i-erythritol, D-mannitol, melibiose, L-rhamnose, D-sorbitol, turanose, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-glucuronic acid, β-hydroxybutyric acid, itaconic acid, DL-lactic acid, succinamic acid, L-alanine, L-alanyl glycine, L-aspartic acid, L-ornithine, L-pyroglutamic acid, L-glutamic acid, γ-aminobutyric acid, urocanic acid, uridine, thymidine, 2-aminoethanol, glycerol and DL-α-glycerol phosphate. Does not oxidize α-cyclodextrin, dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, α-D-lactose, lactulose, maltose, D-mannose, methyl β-D-glucoside, D-psiocose, raffinose, sucrose, trehalose, xylitol, pyruvic acid methyl ester, succinic acid monomethyl ester, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, α-hydroxybutyric acid,

γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, glucuronamide, alaninamide, D-alanine, L-asparagine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-proline, D-serine, L-serine, DL-carnitine, inosine, phenylethylamine, putrescine, 2,3-butanediol, α-D-glucose 1-phosphate or D-glucose 6-phosphate. Resistant to streptomycin and sulfamethoxazole plus trimethoprim, and sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, novobiocin, rifampicin, penicillin G and tetracycline. Predominant fatty acids are summed feature 3 (16:1 $\omega$ 7c and/or iso-15:0 2-OH), 16:0 and 18:1 $\omega$ 7c. The DNA G+C content of the type strain is 63.4 mol%.

The type strain, CS-6<sup>T</sup> (=LMG 24215<sup>T</sup>=BCRC 17729<sup>T</sup>), was isolated from a water sample collected from a spring located inside Nature Valley, Hsinchu County, Taiwan.

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## References

- Chen, W. M., Laevens, S., Lee, T. M., Coenye, T., de Vos, P., Mergeay, M. & Vandamme, P. (2001). *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int J Syst Evol Microbiol* **51**, 1729–1735.
- Chung, Y. C., Kobayashi, T., Kanai, H., Akiba, T. & Kudo, T. (1995). Purification and properties of extracellular amylase from the hyperthermophilic archeon *Thermococcus profundus* DT5432. *Appl Environ Microbiol* **61**, 1502–1506.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- GCG (1995). *Wisconsin Package Version 8.1 Program Manual*. Madison, WI: Genetics Computer Group.
- Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kalmbach, S., Manz, W., Wecke, J. & Szewzyk, U. (1999). *Aquabacterium* gen. nov., with description of *Aquabacterium citratiphilum* sp. nov., *Aquabacterium parvum* sp. nov. and *Aquabacterium commune* sp. nov., three *in situ* dominant bacterial species from the Berlin drinking water system. *Int J Syst Bacteriol* **49**, 769–777.
- Kluge, A. G. & Farris, J. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Lányi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- MacFaddin, J. F. (2000). *Biochemical Tests for the Identification of Medical Bacteria*, 3rd edn. Baltimore: Williams & Wilkins.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Powers, E. M. (1995). Efficacy of the Ryu nonstaining KOH technique for rapidly determining gram reactions of food-borne and waterborne bacteria and yeasts. *Appl Environ Microbiol* **61**, 3756–3758.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for constructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.